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Original Paper

Overexpression of Prolidase Induces Autophagic Death in MCF-7 Breast Cancer Cells

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Key Words
Prolidase • Proline • Autophagy • Collagen biosynthesis • Breast cancer cells MCF-7

Abstract
Background/Aims: Proline availability for proline dehydrogenase/proline oxidase (PRODH/POX) may represent switching mechanism between PRODH/POX-dependent apoptosis and autophagy. The aim of the study was to evaluate the impact of overexpression of prolidase (proline releasing enzyme) on apoptosis/autophagy in breast cancer MCF-7 cells. ***Methods:*** The model of MCF-7 cells with prolidase overexpression (MCF-7^{PL}) was obtained. In order to targeting proline for PRODH/POX–dependent pathways substrate for prolidase, glycyl-proline (GP) was provided and proline utilization for collagen biosynthesis was blocked using 2-methoxyestradiol (MOE). Cell viability was determined using Nucleo-Counter NC-3000. The activity of prolidase was determined by colorimetric assay. DNA, collagen and total protein biosynthesis were determined by radiometric method. Expression of proteins was assessed by Western blot and immunofluorescence bioimaging. Concentration of proline was analyzed by liquid chromatography with mass spectrometry. ***Results:*** Prolidase overexpression in MCF-7^{PL} cells contributed to 10-fold increase in the enzyme activity, 3-fold increase in cytoplasmic proline level and decrease in cell viability and DNA biosynthesis compared to wild type MCF-7 cells. In MCF-7^{PL} cells MOE and GP significantly decreased the number of living cells. MOE inhibited DNA biosynthesis in both cell lines while GP evoked inhibitory effect on the process only in MCF-7^{PL} cells. In both cell lines, MOE or MOE+GP inhibited DNA and collagen biosynthesis. Although GP in MCF-7 cells stimulated collagen biosynthesis, it inhibited the process in MCF-7^{PL} cells. The effects of studied compounds in MCF-7^{PL} cells were accompanied by increase in the expression of Atg7, LC3A/B, Beclin-1, HIF-1α and decrease in the expression

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of PRODH/POX, active caspases-3 and -9. ***Conclusion:*** The data suggest that overexpression of prolidase in MCF-7 cells contributes to increase in intracellular proline concentration and PRODH/POX-dependent autophagic cell death.

Introduction

Prolidase known also as Peptidase D or Iminopeptidase [E.C.3.4.13.9] is a cytoplasmic imido-dipeptidase or imido-tripeptidase [1] that cleaves imido-peptides with C-terminal proline or hydroxyproline [2] (Fig. 1A). The physiologic substrate for prolidase is derived mainly from collagen degradation products and also from other degraded proline-containing proteins, including dietary proteins [3, 4] since intestinal hydrolases do not recognize the tertiary amide bonds [1]. Collagen is the most abundant protein containing imino-bonds. In α1 subunit of type I procollagen (1464 amino acids), proline forms 119 bonds with glycine and in α2 subunit of type I procollagen (1366 amino acids) such a doublet occurs 106 times. Most of proline is hydroxylated in matured collagen. In matured, hydroxylated collagen, unhydroxylated proline in gly-pro doublet occurs 25 times [4]. Prolidase activity was found to recycle proline for collagen re-synthesis and therefore the enzyme plays an important role in regulation of collagen biosynthesis.

Proline oxidase (POX), known also as proline dehydrogenase (PRODH) is flavin-dependent mitochondrial enzyme [5, 6]. It converts proline into Δ1-pyrroline-5-carboxylate (P5C) in mitochondria contributing to maintain redox balance in the cells (Fig. 1B). Free proline bearing reducing potential must be quickly utilized, producing $FADH_2$. On the other hand, conversion of P5C to proline through NADPH/NADH is coupled to pentose phosphate pathway and glucose metabolism [5-8].

During oxidation of proline by PRODH/POX, electrons are transferred to respiratory chain producing ATP or they directly reduce oxygen, producing reactive oxygen species (ROS). In general, PRODH/POX-dependent ATP generation facilitates cell survival [5, 9-11], while ROS induces apoptosis [11-14]. The mechanism for PRODH/POX-dependent apoptosis or survival is unknown.

We hypothesized that critical factor in this switching process is proline availability. The concentration of this amino acid is increased in neoplastic cells [15, 16], however, the mechanism of this phenomen is not understood. Large amount of intracellular proline is derived from collagen degradation products [17]. Extracellular collagen cleaved by metalloproteinases is further degraded intracellularly in lysosomes to free amino acids, except iminodipeptides, that are hydrolyzed in the cytoplasm to amino acids by prolidase. The enzyme plays important role in cytoplasmic proline concentration and regulation of proline-dependent metabolic responses [18, 19]. One of them is proline-dependent inhibition of proteosomal degradation of hypoxia inducible factor (HIF-1α). This transcription factor induces expression of several "pro-survival" genes, as e.g. vascular endothelial growth factor (VEGF), nuclear factor κB (NF-κB), cyclo-oxygenase-2 (COX-2) [18, 20, 21]. Therefore, cytoplasmic proline concentration represents important regulatory mechanism of HIF-dependent responses. The concentration of this amino acid is regulated by proline utilizing processes as collagen biosynthesis and conversion into P5C [5, 8].

The PRODH/POX-dependent functions are mediated by signaling pathways. PRODH/POX affects epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK) and Wnt/β-catenin pathways [22-24]. These processes may be related to PRODH/POX-dependent ROS signaling. Moreover, PRODH/POX expression is regulated by P53 [25-27]. It suggests that PRODH/POX may have cancer suppressor activity. It has been established that PRODH/POX attenuated HIF-1α signaling [28], glycolysis, angiogenesis, metastasis and survival [18]. Therefore, inhibition of HIF-1α by PRODH/POX can play important role in inhibition of cancer cell growth. However, in stress conditions (hypoxia, starvation) PRODH/POX may facilitate pro-survival phenotype of cancer cells [9, 29-32] by AMPK inducing protective autophagy [31].

The aim of this study is identification of PRODH/POX-dependent pathways that are involved in regulation of apoptosis/survival in MCF-7 cells. We considered that prolidase overexpression that contribute to increase in cytoplasmic proline concentration may represent important mechanism for regulation of apoptosis and survival pathways in MCF-7 cells.

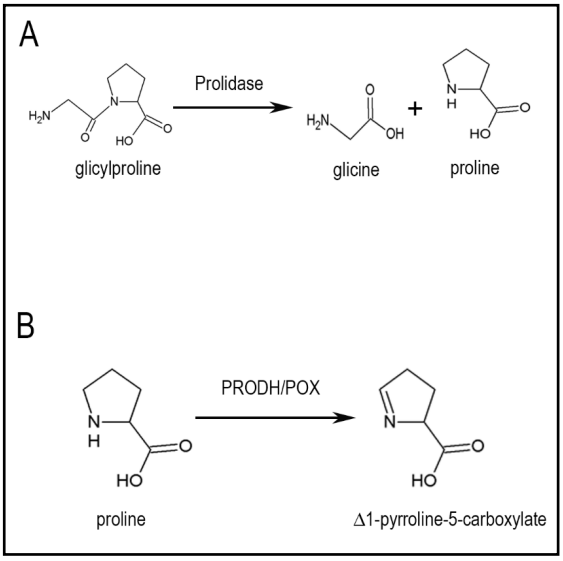

Fig. 1. Reactions catalyzed by prolidase (A) and PRODH/POX (B).

Materials and Methods

Cell lines and culture

Brest cancer cell line MCF-7 was obtained from ATCC (HTB-22, ATCC, Manassas, VA, USA). Prolidase overexpressed MCF-7 (MCF-7^{PL}) cell line was obtained as we described previously [18]. The MCF-7 and MCF-7^{PL} cells were maintained in DMEM and 5% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 50 IU/ml penicillin (Gibco), and 50 µg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere in the presence of 5% CO_2. They were treated for 24 h with GP (17, 22 µg/ml,), MOE (22, 65 µg/ml) or both compounds.

Western-immunoblot analysis

Cell lysates of MCF-7^{PL} and control MCF-7 cells were harvested and subjected to SDS-PAGE in 10% polyacrylamide gel [1h, 125 V, room temperature (RT)]. The protein was transferred to 0.2 µm pore-sized nitrocellulose (wet transfer, 1 h, 100 mA, RT). After the transfer, membranes were blocked with 5% non-fat dry milk in TBS-T (20 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.05% Tween 20, pH 7.4) and incubated with rabbit anti-PEPD (Abcam, Cambridge, United Kingdom), mouse anti-HIF-1α (Becton, Dickinson and Company (B&D), New Jersey, USA), rabbit anti-Atg7 (Cell Signaling (CS), Danvers, USA), rabbit anti-phospho-AMPKα (CS), mouse anti-P53 (B&D), rabbit anti-cleaved-caspase-3 (CS), rabbit anti-cleaved- caspase-9 (CS), mouse anti-P53 (B&D), rabbit anti-cleaved-PARP (CS), diluted 1:1000 in blocking buffer. Then membranes were washed in TBS with 0.05% Tween (TBST) 3 x 15 min and incubated with respective HRP-linked secondary antibody at concentration 1:7500 (Sigma-Aldrich) for 60 min at RT with gentle agitation. After washing in TBS-T (5 × 5 min) membranes were incubated with Amersham ECL Western Blotting Detection Reagent, (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Pictures were taken using BioSpectrum Imaging System UVP (Ultra-Violet Products Ltd, Cambridge, UK).

Cell viability assay

The cell viability was determined using NucleoCounter NC-3000 (ChemoMetec, Copenhagen, Denmark). Prior the experiment MCF-7 and MCF-7^{PL} cells were cultured in six-well plates at 1×10^6 cells/well in 2 ml of growth medium for 48 h. Then, the cells were incubated for 24 h with or without GP, MOE and MOE+GP. After that time the medium was discarded and the cells were rinsed three times with phosphate buffered saline (PBS). The cells were harvested, washed and stained with VitaBright-48 (VB-48) (ChemoMetec), acridine orange (AO) (ChemoMetec), propidium iodide (PI) (ChemoMetec) and analyzed using NC-3000 cell counter.

DNA biosynthesis assay

Proliferation of MCF-7 and MCF-7^{PL} cells was measured by [methyl-^{3}H]-thymidine (Hartman Analytic GmbH, Braunschweig, Germany) incorporation into DNA. Prior the experiment MCF-7 and MCF-7^{PL} cells were cultured in 24-well plate at 1×10^5 cells/well in 1 ml of growth medium. After 48 h the cells were incubated with or without GP, MOE and MOE+GP for 20 h and next with 0.5 µCi/ml of [methyl-^{3}H]-thymidine

for 4 h. The cells were rinsed 3 times with PBS and solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS and 5 ml of scintillation fluid Ultima Gold XR (Perkin Elmer, Waltham, USA). Incorporation of the tracer into DNA was measured by Liquid Scintillation Analyzer Tri-Carb 2810 TR (Perkin Elmer) and calculated using Quanto Smart TM software (Perkin Elmer).

Total protein and collagen biosynthesis

Total protein biosynthesis was measured by incorporation of radioactive proline into proteins. Collagen biosynthesis was measured by incorporation of radioactive proline into proteins digestible by purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky et al. [33]. The cells were cultured in 6-well plates at 1×10^6 cells/well with 2 ml of growth medium. After 48 h, the cells were incubated with 5[^{3}H]-proline (5 μCi/ml), GP, MOE or MOE+GP for 24 h. After isolation of proteins the incorporation of tracer was measured in total proteins and collagenase-digestible proteins. Results are shown as combined values for cell plus medium fractions.

Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. [34]. Protein concentration was measured by the method of Lowry et al. [35]. The enzyme activity was reported as nanomoles of proline released from synthetic substrate (GP), during 1 min and calculated per milligram of supernatant protein of cell homogenate.

Concentration of proline

Samples were analyzed by an HPLC system (1260 Infinity series, Agilent Technologies, Waldbronn, Germany) consisting of a degasser, binary pump, and thermostated autosampler maintained at 4°C connected to an Agilent Technologies QTOF (6530) mass spectrometry detector. Electrospray ionization (ESI) was used as an ion source in positive ionisation mode. Samples (2 μL) were injected onto a HILIC column (Luna HILIC, 100x2.0mm; 3um; Phenomenex) thermostated at 40°C. The system was operated in positive and negative mode at flow rate 1 mL/min with solvent A - water with 10mM ammonium formate and solvent B – acetonitrile/ water (9:1, v:v) with 10mM ammonium formate. Mobile phase was 100% B during 1.5min in isocratic mode. The gradient started in 1.5 min from 100% B to 70% B in 5.5min, then 40% B in 6.0min, maintained 40% B during 1 min and returned to starting conditions in 0.5 min, keeping the re-equilibration until 10 min. The detector operated in full scan mode from 50 to 1000 m/z with a scan rate of 1 scan per second. Accurate mass measurements were obtained by online mass correction to reference masses delivered continuously during analyses. Reference masses at m/z 121.0509 (protonated purine) and m/z 922.0098 [protonated hexakis (1H,1H,3Htetrafluoropropoxy) phosphazine or HP-921]. The capillary voltage was set to 3000V, the gas temperature was 330°C, the nebulizer gas flow rate was 10, 5 L/min. MS TOF parameters were as follows: fragmentor was set to 140V, skimmer 65 V.

Immunofluorescence microscopy

Immunofluorescence analysis was conducted according to BDB Bioimaging protocol, as described previously [36]. Cells grown on a coverslip were fixed with 3, 7% paraformaldehyde and permeabilized with 0.01% Triton. After blocking with 3% foetal bovine serum, the cells were incubated with primary antibodies (anti-beclin-1, anti-cleaved-caspase-3) at dilutions 1:500, and subsequently with FITC Fluor-conjugated secondary antibody. Cells were also incubated with Hoechst to show the cell nucleus. Samples were visualized with a confocal laser scanning microscope (BD Pathway 855 Bioimager) using AttoVision software.

Statistical analysis

In experiments presented on Fig.2 and Fig. 3, the mean values for six assays ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Shapiro-Wilk test and Kolmogorov-Smirnov test. All results have a normal distribution. To assess statistical significance in conducted experiments, one-way ANOVA with Dunnett's multiple comparison test with 99% confidence interval was used (GraphPad PRISM v5.0, GraphPad Software Inc., San Diego, CA). Results were considered significant at $P < 0.001$ level and are denoted by an asterisk (*). Results were presented as the percentage of control values.

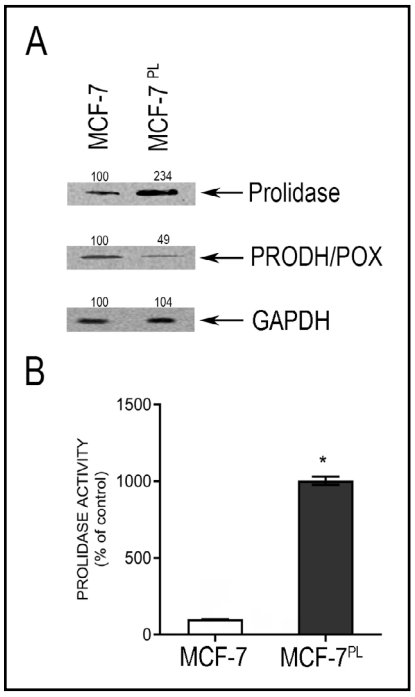

Fig. 2. Prolidase and PRODH/POX expression (A) and prolidase activity (B) in wild type MCF-7 and MCF-7 transfected cells with prolidase cDNA expression vector (MCF-7^{PL}). Representative Western immunoblot for prolidase of control and MCF-7 ^{PL} cells are presented. GAPDH was used as a reference protein. The mean values of bands densitometry were added above appropriated bands. For prolidase activity and Western blot analysis (Supplementary data – see www.cellphysiolbiochem.com) the mean values for 6 assays ± S.D were calculated and submitted to statistical analysis using the ANOVA test, accepting P<0.001 as significant.

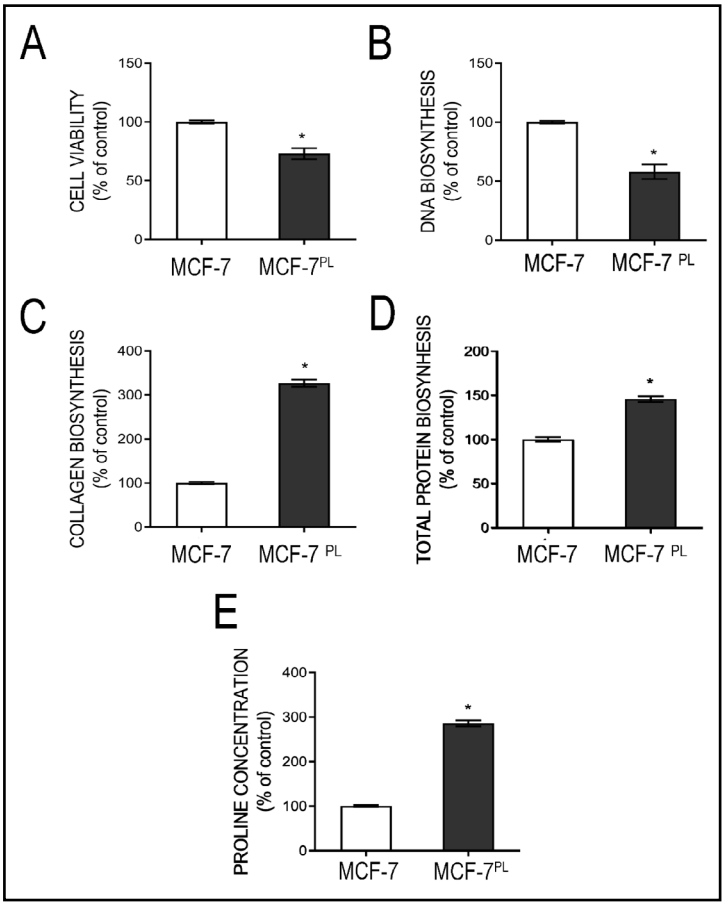

Fig. 3. Cell viability (A), DNA biosynthesis (B), collagen biosynthesis (C), total protein biosynthesis (D) and proline concentration (E), in untreated wild type MCF-7 and MCF-7 transfected cells with prolidase cDNA expression vector (MCF-7 ^{PL}). The mean values for 6 assays ± S.D were calculated and submitted to statistical analysis using the ANOVA test, accepting P<0.001 as significant.

Results

Prolidase overexpression model of MCF-7 cells

The hypothesis that prolidase may play a regulatory role in PRODH/POX-dependent apoptosis/autophagy, led us to establish an experimental model of prolidase overexpression in MCF-7 cells by transfecting the cells with prolidase cDNA expression plasmid. The stably transfected cells showed markedly increased prolidase expression (Fig. 2A), 10-fold increased the enzyme activity (Fig. 2B) and decreased PRODH/POX expression (Fig. 2A), compared to the empty vector transfected cells, considered as a control.

Effect of prolidase overexpression on cell viability, DNA biosynthesis, collagen biosynthesis, total protein biosynthesis and proline concentration in MCF-7 cells

Prolidase overexpression contributed to decrease in cell viability to about 70% of control (Fig. 3A) and DNA biosynthesis to about 55% of control (Fig. 3B). However, in MCF-7^{PL} cells collagen biosynthesis (Fig. 3C), total protein biosynthesis (Fig. 3D) and proline concentration (Fig. 3E) were significantly increased compared to wild-type MCF-7 cells. In these cells collagen biosynthesis and proline concentration were elevated by 3-fold, while total protein biosynthesis only by 50% compared to wild-type MCF-7 cells.

Effect of glycyl-proline (GP), 2-methoxyestradiol (MOE) and MOE + GP on cell viability, DNA biosynthesis, collagen biosynthesis, total protein biosynthesis and proline concentration in MCF-7 and MCF-7^{PL} cells

Considering important role of proline availability for POX-dependent regulation of apoptosis/autophagy, we used GP (a substrate for prolidase in order to increase cytoplasmic level of proline) and MOE (an inhibitor of proline utilization for collagen biosynthesis). To underline the effect of GP, MOE and MOE + GP on studied processes, the values in both control cell lines (untreated) were considered as 100%.

No significant effect of studied compounds (MOE and GP) on the mean percentage of living cells was found in MCF-7 (Fig. 4A). However, in MCF-7^{PL} cells MOE decreased cell survival to about 65% and GP or MOE + GP to about 80% of control (Fig. 4A). MOE inhibited DNA biosynthesis in both cell lines and GP evoked inhibitory effect on the process only in MCF-7^{PL} cells. However, an addition of MOE+GP contributed to inhibition of DNA biosynthesis in both cell lines (Fig. 4B). MOE inhibited collagen biosynthesis in MCF-7 and MCF-7^{PL} cells to about 20% of control. Although, GP in MCF-7 cells stimulated collagen biosynthesis (by about 20%), it inhibited the process in MCF-7^{PL} cells to about 70% of control. However, MOE+GP inhibited collagen biosynthesis in both cell lines, to about 25% of control (Fig. 4C). Similar rate of total protein biosynthesis under the effect of studied compounds was found in wild-type MCF-7 cells while in MCF-7^{PL} cells the inhibitory effects were less pronounced (Fig. 4D). All studied compounds increased proline concentration in both cell lines (Fig. 4E), however, to the higher extent in MCF-7^{PL} cells.

Effect of glycyl-proline (GP), 2-methoxyestradiol (MOE) and MOE + GP on expression of PRODH/POX and apoptosis/survival markers in MCF-7 and MCF-7^{PL} cells

To test the effect of prolidase overexpression on apoptosis/autophagy-inducing pathways we analyzed expression of several markers. For apoptosis we measured expression of cleaved Caspase-3 and Caspase-9, cleaved PARP and P53 while for autophagy AMPK, ATG7 and HIF-1α in GP, MOE and MOE+GP treated and untreated MCF-7 and MCF-7^{PL} cells (Fig. 5).

Expression of autophagy markers AMPK, ATG7, LC3A/B and HIF-1α was increased in MCF-7^{PL} cells, compared to wild type MCF-7, particularly in the presence of MOE or GP (Fig. 5A). In contrast, expression of apoptosis markers, cleaved Caspase-3 and -9, cleaved PARP, as well as P53 was decreased in MCF-7^{PL} cells compared to MCF-7 cells. In fact, apoptosis markers were highly expressed in MCF-7 cells, compared to MCF-7^{PL} cells (Fig. 5A). Although the expression of active caspase-3 and caspase -9 were increased in MOE or GP – treated MCF-7 cells, it was not the case in respect to P53 and active PARP. Under the effect of studied

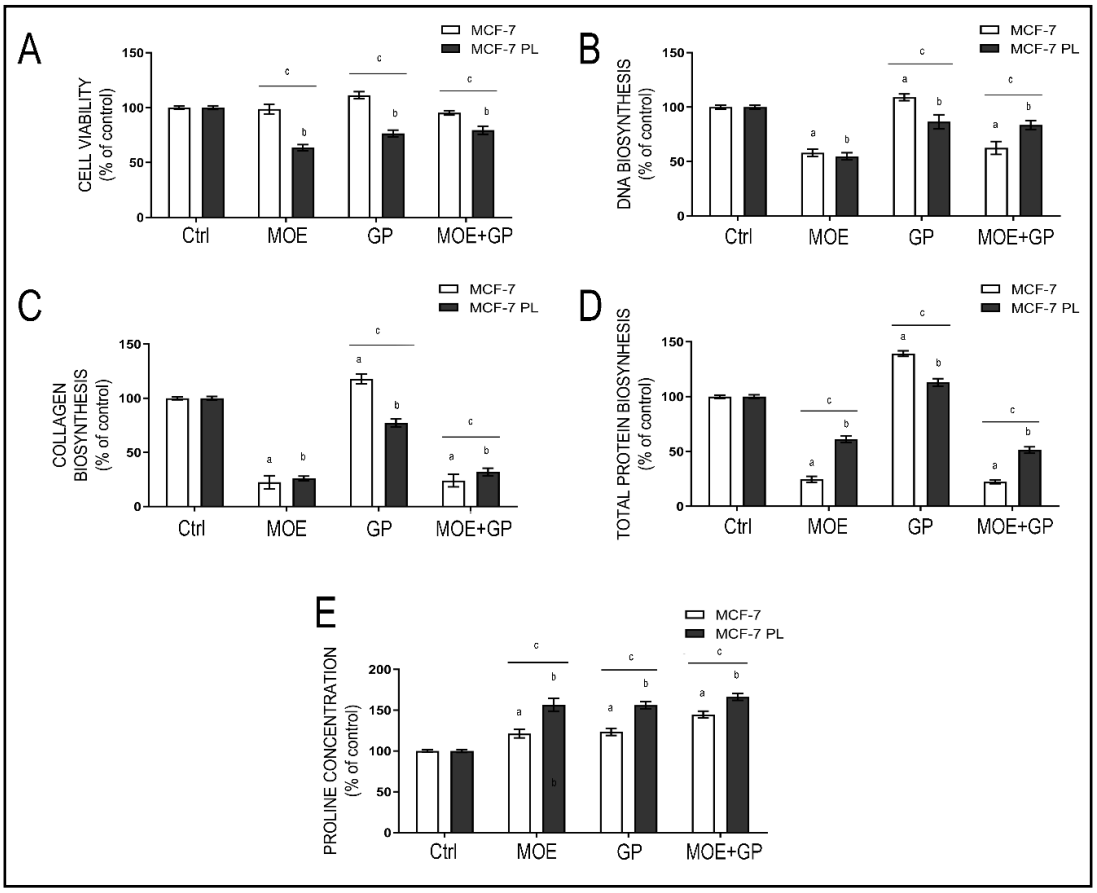

Fig. 4. Cell viability (A), DNA biosynthesis (B), collagen biosynthesis (C), total protein biosynthesis (D) and proline concentration (E) in wild type MCF-7 and MCF-7 transfected with prolidase cDNA expression vector (MCF-7 ^{PL}) untreated (Ctrl) and treated with glycyl-proline (GP), 2-methoxyestradiol (MOE) and MOE + GP. The mean values for 6 assays ± S.D were calculated and submitted to statistical analysis using the ANOVA test, accepting $P<0.001$ as significant. Statistically significant differences are marked as: ^{a} vs. control wild-type MCF-7 cells, ^{b} vs. control MCF-7^{PL} cells, ^{c} wild-type MCF-7 cells vs. MCF-7^{PL} cells after treated by the same substances.

compounds, expression of P53 and active PARP were decreased in MCF-7^{PL} cells, compared to the control cells. Up-regulation of autophagy markers in MCF-7^{PL} cells compared to wild-type MCF-7 cells was confirmed by immunofluorescence bioimaging of Beclin-1. As shown on Fig. 5B, MCF-7^{PL} cells strongly expressed Beclin-1, particularly in the presence of MOE and GP. In contrast, cleaved caspase-3 was strongly expressed in wild-type MCF-7 cells and the effect was augmented by MOE and GP. Although the expression of PRODH/POX was decreased in MCF-7^{PL} cells compared to wild-type MCF-7 cells, all studied compounds had no effect on the process in both cell lines.

Discussion

Although the role of PRODH/POX in activation of apoptosis and autophagy is well established, the mechanism for differential function of the enzyme is unknown. In this report, we suggest that prolidase overexpression in MCF-7 cells (MCF-7^{PL}) induces PRODH/POX-dependent autophagic cell death and the process is determined by proline availability (by application of MOE or GP). In contrast, in wild-type MCF-7 cells increase in proline

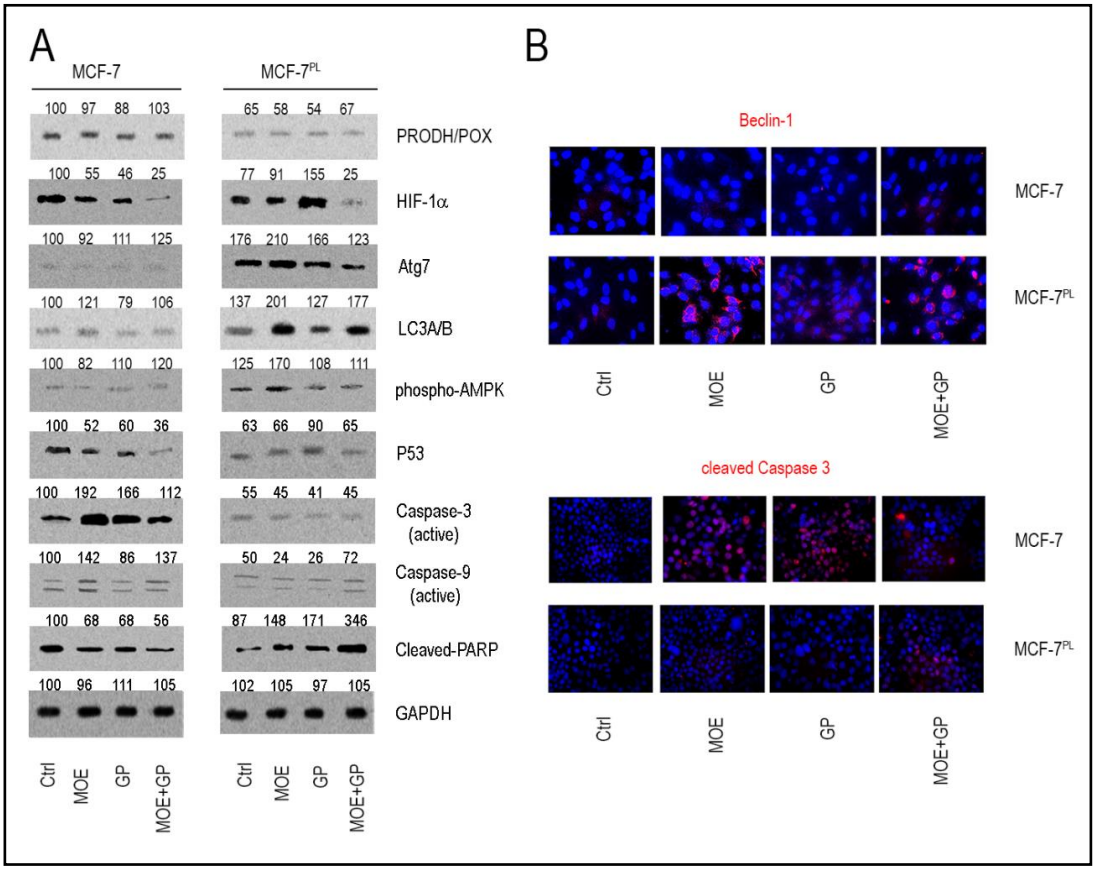

Fig. 5. Western immunoblot (A) for PRODH/POX, autophagy markers (HIF-1α, Atg 7, LC3A/B, phospho-AMPK, beclin-1) and apoptosis markers (p53, cleaved caspase-3 and -9, cleaved PARP) and immunofluorescence analysis (B) for beclin-1 and cleaved Caspase-9 in wild type MCF-7 and MCF-7^{PL} cells untreated (Ctrl) and treated with glycyl-proline (GP), 2-methoxyestradiol (MOE) or MOE + GP in glutamine-free DMEM for 24h. GAPDH was used as a reference protein. The mean values of bands densitometry were added above appropriated bands. All bioimages are included in the Supplementary Material section (see www.cellphysiolbiochem.com).

concentration contributed to pro-apoptotic phenotype as detected by expression of active Caspase-3, -9 and PARP. The data were corroborated by immunofluorescence analysis of Beclin-1, showing up-regulation of Beclin-1 in MCF-7^{PL} cells. However, of special interest is observation that in MCF-7^{PL} cells, increase in autophagy markers was accompanied by decrease in cell viability and DNA biosynthesis, particularly when proline concentration was increased by treatment of the cells with MOE, GP or both. It suggests that in a such condition autophagy contributed to death of large number of cells. The mechanism for the process is known as an autophagic cell death [37, 38]. Whether this is the case in studied condition requires further study. However, based on the interplay between PRODH/POX, proline and proline supporting or proline utilizing processes it provides explanation for the mechanism of PRODH/POX-dependent regulation of apoptosis/autophagy.

Previously we suggested that the switching from autophagic to apoptotic phenotype in MCF-7 cells is mediated by PRODH/POX at proline availability for this enzyme [39]. It is well established that mitochondrial degradation of proline to P5C promotes ROS-dependent apoptosis [40]. Alternatively, when PRODH/POX is down regulated, free cytoplasmic proline is increased stabilizing transcriptional activity of HIF-1 α. This is the case in MCF-7^{PL} cells showing low PRODH/POX expression. Such conditions are considered inflammatory and pro-survival, since HIF-1 α induces expression of COX-2, VEGF, TNF-α, IL-1, NF-κB and other

genes involved in inflammatory response [18]. Therefore, proline availability determined by alternative pathways of proline metabolism may represent molecular interface of cell apoptosis/autophagy.

Large amount of proline is released by prolidase from imidodipeptides. The enzyme activity is regulated by β1-integrin receptor signalling [19] and phosphorylation on threonine/tyrosine residues [41]. However, utilization of intracellular proline is as well as important. Proline considered as a stress molecule can be utilized in cytoplasm by incorporation into collagen [42]. Collagen biosynthesis is therefore considered as a sink for proline contributing to removal of proline reducing potential. Unfortunately, this process promotes tissue cirrhosis [43, 44]. On the other hand, increase in proline level (due to inhibition of PRODH/POX activity) impairs regeneration of oxidizing potential contributing to suppression of cellular metabolism, including collagen biosynthesis [45, 46]. The same mechanism may apply to inhibition of cell proliferation in MCF-7^{PL} cells that is associated with increase in proline level and impairment of redox potential. Therefore, it seems that prolidase activity and PRODH/POX expression may represent molecular inter-face that can switch on and off autophagic or apoptotic mode [47]. We found that in MCF-7^{PL} cells, increase in cytoplasmic proline concentration by MOE or GP contributed to down regulation of PRODH/POX, DNA biosynthesis and cell survival through induction of autophagic mode in these cells.

We considered HIF-1α as on important player in this switching mechanism. In fact, overexpression of prolidase (particularly in the presence of prolidase substrate), contributed to increase in HIF-1 α transcriptional activity [18, 20]. Prolidase-dependent regulation of HIF-1 α expression was also presented in two breast cancer cell lines of different prolidase activity. These data provided evidence that prolidase plays an important role in the autophagic/survival pathways [18]. Such a situation (autophagy) could take place when PRODH/POX expression is low. In fact, low PRODH/POX expression was found in various types of cancer [48]. It could provide conditions for cancer cell survival. However, when PRODH/POX is expressed, it converts proline to P5C contributing to production of glutamate and α-ketoglutaric acid (αKG), that inhibits transcriptional activity of HIF-1. αKG as a co-substrate of prolyl hydroxylase domain (PHD) forming HIF-1α complex contributes to increase in HIF-1α degradation and down-regulation of HIF-1 – dependent gene expression [28, 49]. Therefore, PRODH/POX-dependent down-regulation of HIF-1 signalling may suppress cell cancer invasion (angiogenesis) and growth. In certain circumstances it may act as a pro-survival factor [5, 9, 26, 29-32]. For instance, in stress conditions (e.g. hypoxia, starvation, inflammation) PRODH/POX activation supports tumour growth. Hypoxia and glucose deficiency may also induce directly PRODH/POX expression, through AMPK (AMP-activated protein kinase) and mTOR (the mammalian target of rapamycin) pathways [29, 50]. Its knockdown by rapamycin also up-regulates PRODH/POX [26, 31, 50]. Whether prolidase expression affects these processes requires to be explored.

Another interesting observation is that the differences between MCF-7 and MCF-7^{PL} cells in respect to apoptosis/autophagy phenotype were accompanied by differences in P53 expression. In contrast to MCF-7 cells, expression of P53 is low in MCF-7^{PL} cells. The possible explanation for the functional significance of P53/prolidase correlation comes from studies showing that P53 can be supressed by forming complex with prolidase [51]. In fact, we found that in MCF-7^{PL} cells prolidase expression was elevated providing conditions for sequestration of P53 and suppression of apoptotic pathway. Moreover, inhibition of P53 could contribute to down regulation of PRODH/POX since P53 is known as the most potent transcriptional regulator of PRODH/POX expression [52]. All those processes link prolidase and proline metabolism to apoptosis/autophagy suggesting novel targets for breast cancer therapy.

Conclusion

Our data show that in MCF-7^{PL} cells, autophagy markers are up-regulated and the process is supported by proline availability. Although prolidase overexpression down-regulated

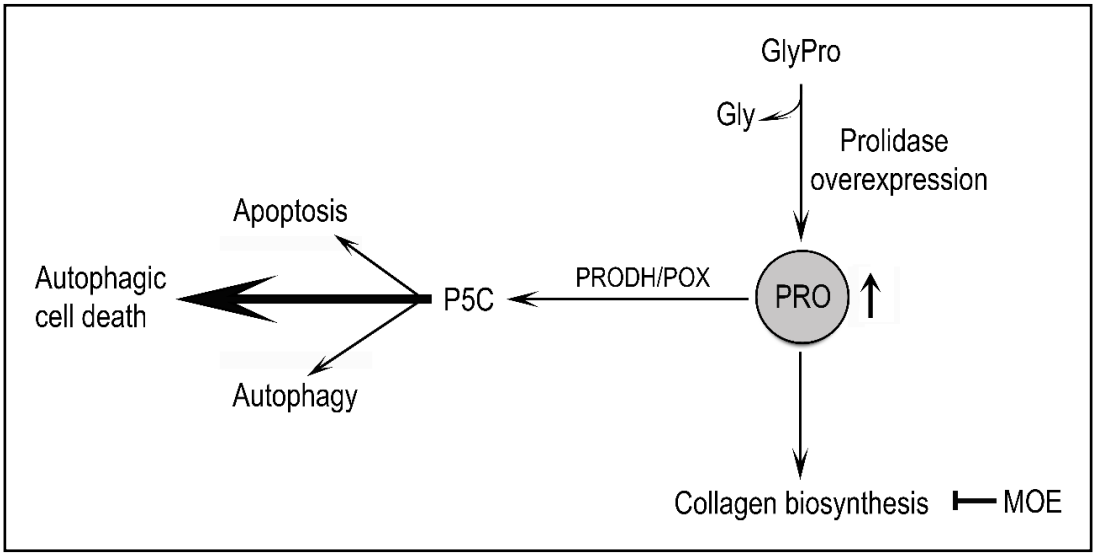

Fig. 6. Prolidase overexpression induces PRODH/POX-dependent autophagic cell death in MCF-7 cells. Gly-Pro – glycyl-proline, substrate for prolidase; MOE – methoxyestradiol, inhibitor of collagen biosynthesis; PRO – proline; P5C – Δ1-pyrroline-5-carboxylate; PRODH/POX – proline dehydrogenase/proline oxidase.

apoptosis markers, it contributed to inhibition of DNA biosynthesis and cell survival. Therefore, we suggest that prolidase overexpression modulate PRODH/POX-dependent autophagic cell death in MCF-7 cells (Fig. 6).

Abbreviations

AMPK (AMP-activated protein kinase); Atg7 (autophagy Related 7); ATP (adenosine triphosphate); cDNA (complementary DNA); COX-2 (cyclooxygenase-2); EGFR (epidermal growth factor receptor); FADH2 (reduced form of flavin adenine dinucleotide); GP (glicyl-proline); HIF-1a (hypoxia inducible factor); IL-1 (interleukin 1); MAPK (mitogen-activated protein kinase); MCF-7 (Human Caucasian breast adenocarcinoma); MCF-7^{PL} (prolidase overexpressed MCF-7); MOE (2-methoxyestradiol); MOE+GP (2-methoxyestradiol and glicyl-proline); mTOR (mammalian target of rapamycin); NADPH/NADH (nicotinamide adenine dinucleotide phosphate/ nicotinamide adenine dinucleotide); NF-κB (nuclear factor κB); P53 (tumor suppressor protein); P5C (Δ1-pyrroline-5-carboxylate); PARP (poly(ADP-ribose) polymerase); PRODH/POX (proline dehydrogenase/proline oxidase); ROS (reactive oxygen species); TNF-α (related apoptosis-inducing ligand α); VEGF (vascular endothelial growth factor); αKG (α-ketoglutaric acid).

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors have read and approved the manuscript. JP, IZ, AS, designed the study concept; IZ, AS, AKLU, JT, AKAZ, JM and TYLH performed the experiments; all the authors analyzed and interpreted the results; IZ, AS, JP drafted and improved the manuscript.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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